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14. ABSTRACT We have examined alterations in inhibitory GABA signaling in mouse models of ASDs as well as in human iPS derived neurons. We had previously demonstrated that the maturation of the reversal potential for GABA (E_{GABA}) is delayed in the Fragile X mouse model. In this project we proposed to determine whether this alteration in the maturation of E_{GABA} was a convergent alterations in ASDs with multiple genetic origins, which would make it an important target pathway in ASDs. Also we proposed to determine whether inhibiting one of the chloride co-transporters that control E_{GABA} could be used as a corrective strategy for the synaptic and circuit disruptions demonstrated in the Fragile X mouse model. We found: 1) that E_{GABA} development was not disrupted in the mouse model of Angelman Syndrome 2) E_{GABA} development was delayed in human induced neurons derived from Fragile X patient fibroblasts 3) Inhibiting the Cl- co-transporters that regulate E_{GABA} by administration of bumetanide to mice rescues synaptic and circuit dysfunction in Fragile X mice.						
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1. **INTRODUCTION:** Autism spectrum disorders (ASDs) are a group of neurodevelopmental disorders with complex and heterogeneous symptom presentation. ASDs can be broadly grouped into syndromic and non-syndromic groups based upon their genetic components. In the case of syndromic ASDs these usually are a facet of the phenotype presenting with another neurodevelopmental disorder of known genetic etiology (e.g. Fragile X syndrome). Whereas non-syndromic autism can be due to sporadic mutations or inherited mutations in genes not associated with other syndromes. While there are an increasing number of variants associated with ASDs in genetic loci with a diverse range of functions, increasingly there is accumulating evidence that the disruptions at both the synaptic and circuit level that contribute to the autism phenotype are common in ASDs with diverse genetic origins. Our laboratory has been examining alterations in inhibitory GABA signaling in mouse models of ASDs as well as in human iPS derived neurons. We had previously demonstrated that the maturation of the reversal potential for GABA (E_{GABA}) is delayed in the Fragile X mouse model. This sets the “strength” and polarity of GABA and during the early developmental period can have a trophic effect on the development of neurons and synapses. In this project we proposed to determine whether this alteration in the maturation of E_{GABA} was a convergent alterations in ASDs with multiple genetic origins which would make it an important target pathway in ASDs. Also we proposed to determine whether inhibiting one of the chloride co-transporters that control E_{GABA} could be used as a corrective strategy for the synaptic and circuit disruptions demonstrated in the Fragile X mouse model. Therefore the broad goals of the proposal were to 1) determine whether altered E_{GABA} during development was also present the mouse model of Angelman Syndrome 2) Whether E_{GABA} alterations are also found in human induced neurons derived from Fragile X patient fibroblasts 3) whether targeting the Cl⁻ transporters that control GABA polarity can rescue the synaptic and circuit dysfunction in the mouse model of Fragile X.

The Approved Statement of Work was broken down into two Specific Aims:

Specific Aim 1 was to define the alterations in chloride homeostasis in cortical neurons of mouse models of autism and induced neurons from human iPS cell lines. This Specific Aim was to be completed in the first 12 months of the award and the results are outlined below in the accomplishment section.

Specific Aim 2 was to determine whether rectifying the altered chloride homeostasis in juvenile neurons can reverse the cellular substrates of cortical hyperexcitability. This aim was to be completed in the second year of the project, and the results from this are also outlined below.

2. **KEYWORDS:** Autism Spectrum Disorders, Fragile X Syndrome Angelman Syndrome, GABA, synapse, critical period, E_{GABA} , NKCC1, bumetanide, iPS cell, induced neuron.

3. **ACCOMPLISHMENTS:**

We will outline the accomplishments in each of the specific aims which were split into several subtasks to be completed over the 2 year period of the award. Based on the Approved Statement of Work, the objectives for the first year were to complete the Major Task in Specific Aim 1. This was to characterize the chloride reversal potential in cortical neurons from the mouse model of Angelman Syndrome ($Ube3a^{m-/p+}$) and from human derived induced neurons from patient iPS cells.

Major goals and objectives:

Major goals were to characterize the development of E_{GABA} (GABA reversal potential) in cortical neurons in the mouse model of Angelman Syndrome and in human induced neurons derived from patient iPS cells. The reversal potential for GABA is an important indicator of maturation of neurons. During early cortical development, GABA_A receptor mediated responses in neurons are excitatory because the reversal potential for Cl⁻ ions is more depolarized than the resting membrane potential of the cell. At the close of the critical period there is a shift in E_{GABA} to more hyperpolarized potentials, and GABA transmission produces mature hyperpolarizing responses. This switch is known to be delayed in several neurodevelopmental disorders including Fragile X Syndrome, some forms of syndromic and non-syndromic autism, and some childhood epilepsies. Altered E_{GABA} itself may result in effects on the development of synaptic connections especially during early critical or vulnerable periods. E_{GABA} matures due to the upregulation of the K⁺-Cl⁻ co-transporter (KCC2) that extrudes Cl⁻ out of neurons, and a coordinated downregulation of the juvenile Na⁺-K⁺-Cl⁻ co-transporter (NKCC1) that maintains the high internal Cl⁻ concentration in immature neurons. The early depolarizing GABA response plays a trophic role in cellular proliferation and synaptic maturation throughout the neonatal brain and a developmental delay in the maturation of E_{GABA} in the cerebral cortex would contribute to a disruption of synaptogenesis and circuit assembly (common features of ASDs).

Major Goal 1: Characterize the development of chloride reversal in mouse models of autism and induced neurons from patient iPSCs:

Subtask 1: **Examine GABA mediated currents in cortex of *Ube3a*^{m-/p+} mice.** This subtask was due for completion between months 1-6 of the award. This goal has been largely addressed. We set up a colony of

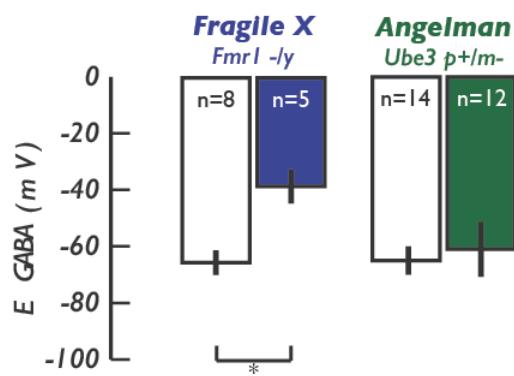
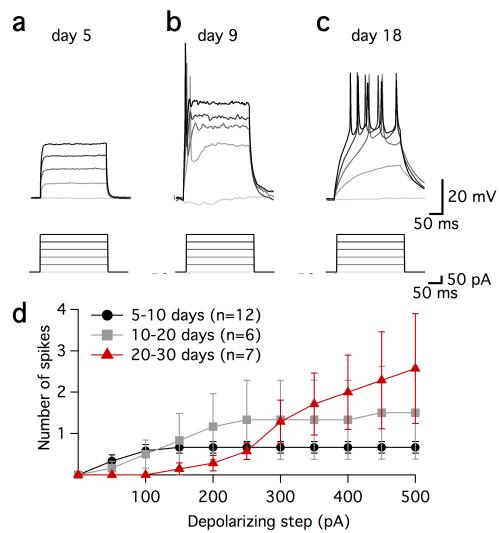


Figure 1 E_{GABA} is not disrupted in Angelman mice at P10. GABA reversal potential was recorded in individual neurons from Fragile X and Angelman mouse models. E_{GABA} was more depolarized in FRX neurons compared to controls but not in cortical neurons from Angelman mice.

Angelman mice and have recorded from layer IV neurons in somatosensory cortex. In normal mice E_{GABA} matures over the course of the critical period during the first week after birth. Therefore we made single cell recordings from individual neurons in cortical brain slices

from animals ranging from P5 to P10. GABA input to the recorded neurons was stimulated by extracellular stimulation in the cortex and GABA responses measured while voltage clamping neurons at various holding potentials. This enabled us to construct a current-voltage curve to GABA responses and measure the reversal potential for GABA as the intersection of the linear fit of the data points with the x-axis. The results are shown in Figure 1. Making comparison between mutant *Ube3a*^{m-/p+} and littermate controls *Ube3a*^{m+/p+} we found no difference in E_{GABA} between the two groups at any developmental stage during the critical period. Figure 1 illustrates the population data from recordings at one developmental timepoint (P10) at the close of the critical period in layer IV. At this timepoint E_{GABA} is normally close to its hyperpolarized mature value. However in Fragile X mice E_{GABA} remains depolarized at this point (Figure 1). When we performed similar experiments in the Angelman mouse model we found no evidence that E_{GABA} was slow to mature. Figure 1 illustrates the population data from one developmental timepoint (P10). This demonstrates that our initial hypothesis that would be a shared endophenotype across the two mouse models was not correct. Therefore we have completed this section and will not pursue this line of enquiry further.

Subtask 2/3. **Develop and maintain induced neurons derived from human patient fibroblasts.** This subtask was due between months 1-24. The neurons differentiated from iPS cells were to be characterized functionally

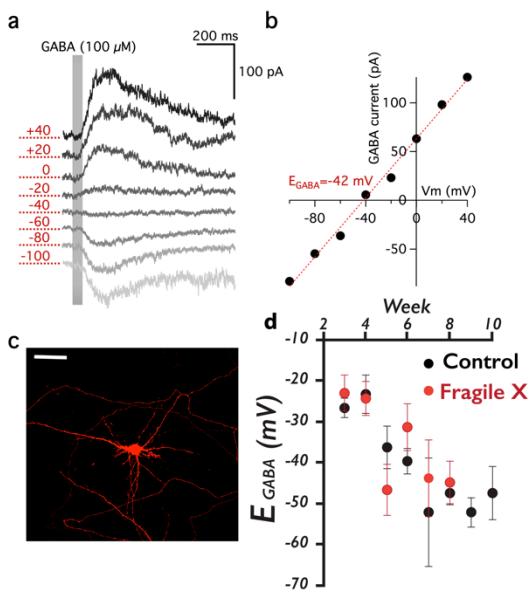


in the other sections of this proposal. After some initial problems with obtaining the appropriate lines from patients (which are outlined below) we have now successfully established several patient lines that have been analyzed using electrophysiological recordings in our laboratory. We used forced expression of NGN2 to differentiate the iPS cells into neurons and have been able to maintain these *in vitro* for over 6 months. To ensure that the cells differentiated from iPS cells are in fact neurons, we performed patch clamp recordings from individual neurons to determine whether

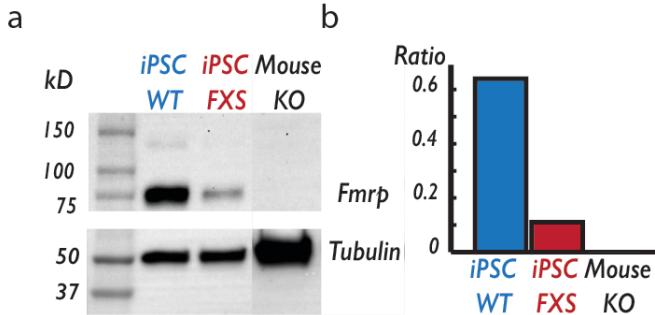
Figure 2: Development of firing properties of human derived iNs (a-c) Representative traces response to depolarizing current injection steps at 3 timepoints after differentiation. (d) Input – output curve of number of spikes with increasing current injection from all recordings of iNs at different timepoints after differentiation.

they had the functional characteristics of neurons. Recording at different timepoints after differentiation we found that recorded cells displayed suprathreshold voltage response, firing action potential, in response to current injection after a number of days of maturation in the culture (Figure 2). These results give us confidence that the culture and differentiation procedures are working, and are producing induced neurons.

Subtask 4. **Examine GABA reversal potentials in induced neurons.** The primary goal of this section of research was to determine whether E_{GABA} matured normally in neurons derived from fragile X patient iPS cells compared to those from healthy patient cells. We therefore made Gramicidin perforated patch clamp recordings



illustrate a single recording from a cell 3 weeks in culture. GABA responses (Fig 3a) reverse around -42 mV (Fig 3b) in this recording. Fig 3c shows the morphology of the induced neuron that is filled with a fluorescent Alexa dye showing extensive dendritic arbors. Finally E_{GABA} recorded at various times after differentiation allowed us to determine the maturation of E_{GABA} (Fig 3d). We expected that there would be a delay in this maturation in human derived neurons from FRX patients as has been demonstrated in the mouse model of the disorder. However our initial recordings did not reveal a difference between the two lines (healthy patient and FRX patient) we examined (Figure 3d).



there was still significant FMRP expression observable in the iPS cells (Figure 4). This was likely because the patient was mosaic. Because of this unforeseen problem with the initial cell line we obtained several additional cell lines from the original source (CHOC bank) as well as from other investigators in the field. We have differentiated these neurons (after first determining that FMRP expression was eliminated) and have made single cell gramicidin perforated patch recordings from neurons in vitro to establish E_{GABA} (as before). By recording from cultures at each week after differentiation (starting at week 2) and measuring E_{GABA} in two cell lines (one a normal control and one from a fragile X patient) we determined that the rate of maturation in the Fragile X patient line was significantly slower (Figure 5). So far these results demonstrate that human induced neurons have a similar endophenotype to those in mouse cortex. This experiment is almost complete (requiring a number of technical replicates) and we expect to publish some of these results in the coming months.

The second specific aim of the project was to determine whether the altered development of E_{GABA} in the cortex of the mouse model of FRX could be rectified by inhibiting the juvenile Cl- transporter NKCC1.

Major Task 2: Determine whether inhibition of chloride transporter *in vitro* shifts chloride reversal.

Subtask 1: Determine whether chronic or acute bumetanide administration hyperpolarizes the GABA reversal potential in Fmr1 ko mice. This subtask was scheduled for months 12-15 of the award. As we have shown

from neurons derived from healthy or patient iPS

Figure 3: GABA currents evoked from iNs derived from FXS patient iPS cells (a) Representative GABA-mediated currents at 3 weeks in vitro (perforated patch) (b) Current/voltage relationship for GABA currents for this experiment. GABA reversal potential is relatively depolarized (-42mV) at this stage in vitro. (c) Image of biocytin-filled iN after 6 weeks in vitro. Calibration: 50μm (d) E_{GABA} determined in iNs at different times in vitro for all recordings from derived from FRX and healthy control cells.

cells and established how E_{GABA} develops over time in culture. We found that E_{GABA} developed very slowly towards its mature value over six months (very different to rodent neurons). During the second week after differentiation E_{GABA} in induced neurons is very depolarized and becomes less so over the next few weeks. Figure 3a and b

Figure 4 Western blot analysis of iPS cells (a) Representative blot probed with anti-FMRP. FMRP is expressed in both FRX patient and healthy control lines (b) analysis of relative expression.

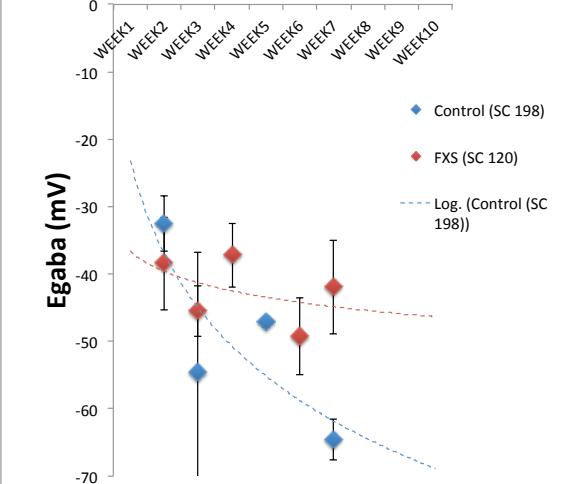
Upon further investigation we found that the patient cell line we had received, although it came from a patient with a full mutation,

previously E_{GABA} is relatively depolarized at the close of the cortical critical period at P10 in fragile X mice and there is a correlated upregulation of NKCC1. Therefore we hypothesized that inhibiting NKCC1 would rectify this deficit. Newborn pups were dosed with bumetanide (once daily I.P.) between P0-P10 after which they were

Figure 5 Time-course of E_{GABA} maturation in human induced neurons in vitro
Recordings were made from neurons induced from iPS cells at several timepoints after differentiation. Cells from two originating clones (SC 198 – normal control, SC 120 – Fragile X patient) were used. The rate of maturation of E_{GABA} is significantly delayed with more depolarized E_{GABA} at later timepoints in culture

sacrificed and recordings made from layer IV neurons in the cortex (Figure 6). We found that there was a significant difference in vehicle treated groups between the ko and littermate controls, confirming what we had previously published (Figure 6B-D). In the bumetanide treated groups we found a significant effect of the drug in the ko which hyperpolarized E_{GABA} to the wildtype level (Figure 6B-D). Figure 6 illustrates these recordings. For the group administered bumetanide for 10 days followed there was a significant hyperpolarization of E_{GABA} (Figure 6D). Moreover we also administered bumetanide to a second group of mice for nine days followed by a one day washout of drug. In this case too we found that E_{GABA} was hyperpolarized to the wildtype values in *Fmr1* ko mice (Figure 6E yellow symbols). However if bumetanide was administered for just a single day followed by recording we found that E_{GABA} was more depolarized in the ko mice compared to their littermate controls at P10 (Figure 6E green symbols). Taken together these experiments demonstrate that systemic administration of an NKCC1 inhibitor can rectify E_{GABA}

Weeks since Dox in culture (Day 0)



in the cortex of Fragile X mice.

Subtask 2 was to determine whether hypersynchrony existed in the cortex of *Ube3a*^{m-/p+} mice, however because we had determined in experiments in Aim 1 that this mouse model of Angelman does not have an overlapping phenotype, these experiments were not pursued. Subtask 3 was pursued in the final year of the award. The goal of this subtask was to determine whether correcting E_{GABA} during the critical period

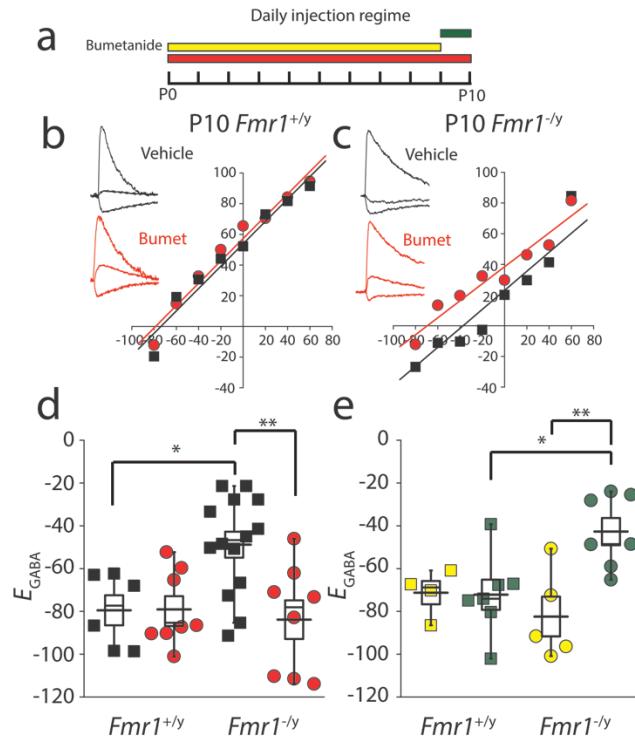


Figure 6 Bumetanide administration rectifies chloride homeostasis. (a)

Dosing regime for the mice. (b) Representative recordings and current voltage plots of GABA responses in vehicle and bumetanide treated WT and (c) knockout mice (d) Summary of all recordings demonstrating that E_{GABA} is relatively depolarized in vehicle treated knockout mice but that this is reversed by bumetanide treatment (e) Results from all experiments in 9 day treated and 1 day treated mice. 9 day treatment rectifies E_{GABA} whereas 1 day treatment does not.

as we have shown in subtask 1 (Figure 6), would also rectify the synaptic plasticity phenotype in Fragile X mice. We had previously demonstrated that at the close of

the critical period, excitatory synapses remain plastic in *Fmr1* KO mice at a postnatal time when there is no long term potentiation (LTP) in WT mice. This developmental plasticity is dependent on NMDARs and correlates with the fraction of silent synapses. We tested whether treating mice with bumetanide, which corrects

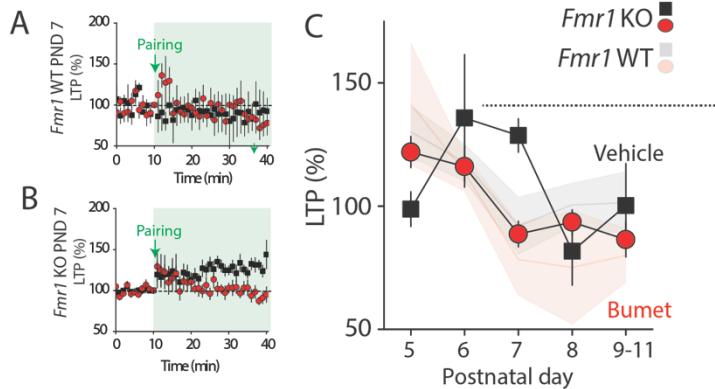


Figure 7 Timecourse of LTP is rescued in *Fmr1* ko mice by administration of bumetanide **(A)** Grouped time course of thalamocortical LTP in vehicle or bumetanide treated *Fmr1* WT and **(B)** *Fmr1* KO mice at PND 7. All values are mean \pm s.e.m. **(C)** Temporal progression of thalamocortical LTP from grouped data of vehicle and bumetanide treated *Fmr1* KO mice at each postnatal day between P5 and P11. Opaque graphs are LTP progression in *Fmr1* WT in vehicle and bumetanide. All values are mean \pm s.e.m * $p < 0.05$ One way ANOVA.

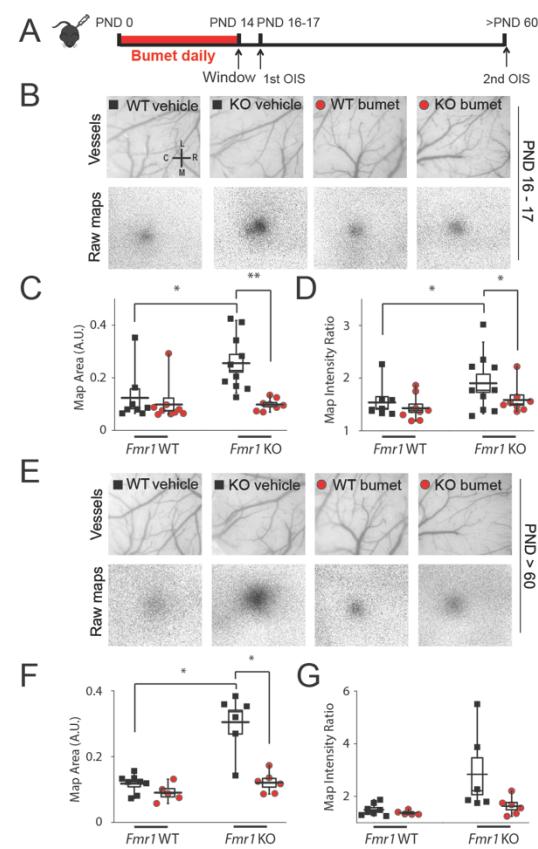
normal time-course of LTP was disrupted over this period peaking abnormally at a later PND (Figure 7C). However, bumetanide treatment in *Fmr1* KO mice corrected this profile so that LTP was robust at the youngest ages (PND5) but disappeared by PND 7 (Figure 7C). As we had observed in other experiments in *Fmr1* WT mice, there was no effect of bumetanide on LTP measured at any age when compared to vehicle group (Figure 7C, opaque lines). These experiments are the first to confirm that correcting EGABA with bumetanide treatment can correct the development of synaptic plasticity over the course of the critical period. These results are part of a manuscript that has been submitted for review.

Subtask 4 of this Aim was to determine whether chronic bumetanide treatment during critical period development could reverse the circuit hyperactivity in the somatosensory

Figure 8 Long lasting correction of cortical whisker evoked responses after critical period treatment with bumetanide

(A) Schematic representation of time-course of OIS experiments. **(B)** Top: vasculature of S1 somatosensory cortex of PND 16-17 *Fmr1* WT and *Fmr1* KO mice; Bottom: corresponding cortical excitation maps collected during D2 whisker stimulation. **(C)** Map area measured in PND 16-17 *Fmr1* WT and *Fmr1* KO mice in vehicle (black) and bumetanide (red) **(D)** Map intensity ratio measured in PND 16-17 mice **(E)** Vasculature and cortical excitation maps for mice PND > 60 **(F)** Map area measured in mice **(G)** Map intensity ratio measured in mature mice. * $p < 0.05$ ** $p < 0.001$ two way ANOVA.

E_{GABA} , can also correct the normal developmental plasticity in layer IV synapses. Gramicidin perforated patch voltage-clamp recordings were made from mice at PND 7 and LTP was induced by a pairing of repetitive presynaptic thalamic activity and postsynaptic depolarization. In both vehicle- and bumetanide-treated *Fmr1* WT mice, no LTP was observed at this age (Figure 7A). In contrast, there was a significant potentiation of the EPSC 30-40 minutes after LTP induction in vehicle-treated *Fmr1* KO mice (Figure 7B, black symbols); this was completely absent in the bumetanide-treated *Fmr1* KO group, consistent with a normalization of synapses (Figure 7B, red symbols). To fully determine the effect of bumetanide on LTP over the course of the critical period we made further recordings from mice at each postnatal day (PND) 5-11. As we previously demonstrated for *Fmr1* mutant mice, we again found that, in vehicle-treated *Fmr1* KO mice, the



cortex that is observed in Fragile X mice. Prior work has demonstrated that there is a heightened response in the adult somatosensory cortex of *Fmr1* KO mice to stimulation of the whiskers. We used optical imaging of intrinsic signals (OIS) to measure the cortical response to single whisker stimulation in order to determine whether sensory responses were affected by chronic bumetanide treatment during the critical period. *Fmr1* KO and littermate *Fmr1* WT mice were administered bumetanide or vehicle during the first two postnatal weeks (daily from PND 0 to PND 14)(Figure 8A). At P14 mice underwent surgeries to implant cranial windows over the somatosensory cortex and imaging was first performed at PND 16 & 17 and then again at 2 months of age. The sensory representation map evoked by high frequency (100Hz, 1.5s) stimulation of the D2 whisker was measured in all the groups. At PND16/17, the vehicle-treated *Fmr1* KO group had a significantly larger cortical response (thresholded area), and higher map pixel intensity ratio (ratio of maximum map pixel intensity to background pixel intensity) compared to vehicle-treated *Fmr1* WT controls (Figure 8 B-D). In the bumetanide-treated *Fmr1* KO group the cortical whisker response was significantly reduced and indistinguishable from that of either the vehicle- or drug-treated WT animals (Figure 8 B-D). Therefore, the enhanced sensory response in juvenile *Fmr1* KO mice can be normalized by bumetanide treatment. To determine whether drug administration during the critical period has long lasting effects on cortical responses, we also measured the whisker-evoked intrinsic signals in the same animals at 2 months of age. Vehicle-treated adult *Fmr1* KO mice also had abnormally large cortical representations of whisker responses in comparison to the vehicle-treated *Fmr1* WT mice (Figure 8 E-G). However, in the group treated with bumetanide during the critical period the OIS neural activity map was rescued in the KO and was no different from the WT groups (map area WT bumetanide vs KO bumetanide, $p > 0.05$). Therefore, bumetanide treatment during the early cortical critical period has a long lasting effect on correcting the circuit response to sensory whisker stimulation confirming our hypothesis for this aim. These experiments are included in a manuscript that is currently under consideration for publication. This completes all the major goals of this project.

Training and professional development:

The primary goals of this project were not training or professional development. However postdocs who have contributed to the experiments have been trained in several new techniques that were not previously available in our laboratory. Primarily these have involved techniques around the development and maintenance of iPS cells and induced neurons (From the iPS cell core at Northwestern) and also the performance of in vivo OIS studies (trained by our collaborator at UCLA).

Dissemination of results:

Data generated from these experiments has been presented at several scientific meetings including the Gordon Conference on Fragile X and Autism related Disorders, the Society for Neuroscience annual meeting, and the Federation of European Neurosciences meeting. Additional presentations are planned at the Winter Conference for Brain Research in Feb 2017. Data from this project has also been included in a manuscript that is currently submitted and under consideration at a peer reviewed journal. A second article including the iPS cell data is being prepared for submission early next year.

Plans for the next reporting period:

This is the final report "Nothing to report"

4. IMPACT:

The principal impact of this work will be in the field of autism research and moreover in the field of Fragile X syndrome. Our findings that bumetanide treatment during an early critical period in the mouse model can correct alterations at the synaptic, circuit and higher network level will likely have a large impact in developing new treatment strategies. We think that this high impact will potentially allow us to publish this research in a top tier journal which will increase the visibility of the results and therefore increase the potential impact in the field. A second impact is the establishment of functionally characterized human neurons, which will be made available to the field for further research.

5. CHANGES/PROBLEMS:

The only changes have been the use of different cell lines to create induced neurons. The original iPS cells came from a mosaic patient with a full mutation but still expressed FMRP protein. While this problem delayed our experiments, we still were able to get useful data and established all the techniques required for handling and differentiating these cells. The second cell line we have characterized has demonstrated a strong electrophysiological phenotype (delayed E_{GABA} maturation)

No other changes in approach or goals to report and no changes in expenditures.

6. PRODUCTS:

He Q, Arroyo E, Smukowski S, Piochon C, Savas J, Portera-Cailliau C and Contractor A Critical Period Inhibition of NKCC1 restores Synapse Development and Tactile Response Maps in Fragile X Mice *Under Review*

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Anis Contractor
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID): XXXXXX
Nearest person month worked: 1

Contribution to Project: Dr Contractor has led the project and has been responsible for overall administration and direction. He has been primarily responsible for ensuring that there has been a successful and productive collaboration with members of the team such that the goals of the study are met. He has provided his expertise in studying synapses and supervise the experiments of the postdoctoral fellows.

Funding Support: None (Complete only if the funding support is provided from other than this award).

Name: John Kessler
Project Role: Co-Principal Investigator
Researcher Identifier (e.g. ORCID ID): XXXXXX
Nearest person month worked: 1

Contribution to Project: Dr Kessler has been primarily overseeing and supervising the differentiation and maintenance of iPS cells

Funding Support: None (Complete only if the funding support is provided from other than this award).

Name: Toshihiro Nomura
Project Role: Senior Research Associate
Researcher Identifier (e.g. ORCID ID): XXXXXX
Nearest person month worked: 10

Contribution to Project: Dr Nomura has primarily been involved on performing patch clamp recording from mouse cortical slices (Fmr1 ko). He has performed the recordings, analyzed the data and maintained mouse colonies.

Funding Support: None (Complete only if the funding support is provided from other than this award).

Name: Tammy McGuire
Project Role: Senior Research Technologist
Researcher Identifier (e.g. ORCID ID): XXXXXX
Nearest person month worked: 2

Contribution to Project: Ms McGuire's primary role has been to work with culturing the iPS cells and induced neurons

Funding Support: None (Complete only if the funding support is provided from other than this award).

Name: Claire Piochon
Project Role: Senior Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID): XXXXXX
Nearest person month worked: 12

Contribution to Project: Dr Piochon has performed all the recordings from induced neurons as well as recording from the Angelman mice. She has also contributed to recording from drug treated Fmr1 ko mice.

Funding Support: None (Complete only if the funding support is provided from other than this award).

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES: None